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Numerical Chromosomal Aberrations in Thyroid Tumors Detected by Double Fluorescence In Situ Hybridization

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Double fluorescence in situ hybridization with DNA probes specific for the (peri)centromeric regions of chromosomes 3, 7, 9, 11, 12, 18, and X was performed on fresh isolated nuclei and frozen tissue sections prepared from 2 nodular hyperplasias, 2 adenomas, and 7 papillary carcinomas of the thyroid in order to detect numerical chromosomal changes. Numerical chromosomal aberrations were found in all malignant specimens examined. A consistent presence of at least two trisomies was detected in most cases, especially in the follicular variant specimens; the highest degree of trisomy was observed for chromosome 12. Isolated monosomies of moderate degree for different chromosomes were found in 1 adenoma and 2 papillary carcinomas. Severe monosomy of chromosome 9 was the only significant feature observed in the single metastatic papillary carcinoma. *Genes Chrom Cancer 9:180–185 (1994).* © 1994 Wiley-Liss, Inc.

INTRODUCTION

The genetic changes in tumors are receiving growing attention. Cytogenetic analysis of hemopoietic malignancies has revealed several specific structural as well as numerical chromosome aberrations (Croce, 1986). In solid tumors, knowledge about specific cytogenetic changes is far less. Both the low mitotic index of most solid neoplasms and the complexity of their chromosomal changes make it difficult to perform a correct cytogenetic analysis by means of the conventional banding techniques (Mitelman 1983; Heim and Mitelman, 1987, 1989; Sandberg and Turc-Carel, 1987; Sandberg, 1990).

In situ hybridization (ISH) circumvents some of the problems. Non-isotopic ISH, fluorescent or not, can be used to detect numerical chromosome disorders in interphase nuclei also, obviating the need to culture the cells and avoiding interpretation problems with changes emerging in vitro. Chromosome-specific, repetitive alpha-satellite DNA probes can be easily hybridized (Willard and Waye, 1987) to interphase nuclei. Such interphase cytogenetics (Cremer et al., 1986) has been used to detect chromosomal aberrations in neurologic tumors (Cremer et al., 1988a,b; Arnoldus et al., 1991), breast carcinomas (Devilee et al., 1988), urinary bladder carcinomas (Hopman et al., 1988, 1991), hemopoietic malignancies (Van Dekken et al., 1989; Anastasi et al., 1990; Ried et al., 1992), atypical germ cells (Walt et al., 1989), and stomach adenocarcinomas (Van Dekken et al., 1990a).

In thyroid tumors, little is known about specific chromosomal changes, in spite of the fact that cytogenetic studies of in vitro cultures have been performed by several groups (Wurster-Hill et al., 1986; Butler et al., 1987; Mark et al., 1987; Tanaka et al., 1987; Antonini et al., 1989; Bondeson et al., 1989; Olah et al., 1990; van den Berg et al., 1990, 1991). We present the results of fluorescence ISH (FISH) studies of numerical chromosomal changes in 9 thyroid neoplasms: 2 adenomas and 7 papillary carcinomas.

MATERIALS AND METHODS

Samples

Eleven samples representative of different thyroid lesions, and adjacent normal tissue when possible, were selected at the time of the preoperative pathological examination (see Table 1 for clinical and histological data). Each specimen was divided into two parts: One was snap frozen in alpha-methylbutane immediately after the surgical examination and stored at −70°C until used, and the second was treated for routine histological procedures.

Fresh Isolated Nuclei

Nuclear suspensions were obtained by mechanical disaggregation of the frozen samples as described by Arnoldus et al. (1991). Briefly, the frozen thyroid tissue was disaggregated using a Potter–Elvenjem homogenizer. The suspension

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TABLE 1. Sex and Age of Patients and Main Pathologic Features of Thyroid Samples Examined

Case	Sex/age (years)	Histopathologic diagnosis	Other features
1	F/43	Nodular hyperplasia	> size of thyroid
2	F/44	Nodular hyperplasia	> size of thyroid
3	F/22	Follicular adenoma	Size: 0.6 × 7 × 7 cm
4	F/41	Hürthle cell adenoma	Size: 1.3 × 1.2 × 1.2 cm
5	F/60	Papillary carcinoma, follicular variant	
6	M/11	Papillary carcinoma, follicular variant	
7	M/45	Papillary carcinoma, follicular variant	
8	M/17	Papillary carcinoma, follicular variant	
9	F/22	Papillary carcinoma, classical type	
10	F/51	Papillary carcinoma, classical type	Partly encapsulated
11	F/66	Papillary carcinoma, classical type	Multifocal, 7/18 metastatic lymph nodes + lung metastasis

was fixed in 1% acid-free formaldehyde and centrifuged for 10 min. The pellet was dissolved in lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 10 mM EDTA, pH 7.4), centrifuged, and resuspended in 0.5% pepsin (Sigma). The resulting suspension was filtered through a nylon filter (diameter 80 μm). After fixation (70% ethanol), the nuclei were centrifuged onto glass slides for 5 min at 135 g and dehydrated through an ethanol series (70%, 90%, 100%).

Frozen Tissue Sections

In order to verify whether FISH on tissue sections could give useful information, we hybridized DNA probes, arbitrarily selected but specific for chromosomes 7 and 11, to thyroid sections. In 4 cases (1 nodular hyperplasia, 1 follicular adenoma, and 2 papillary carcinomas, follicular variant), double FISH was performed on isolated nuclei as well as on tissue sections. In these instances, frozen sections were cut (7–10 μm thick) and immediately fixed in 4% paraformaldehyde for 10 min and dehydrated in an ethanol series (70%, 90%, 100%; Taruscio et al., 1993).

DNA Probes

The DNA probes, specific for the (peri)centromeric regions of chromosomes 3 (p3–5), 7 (p7tet), 9 (pMR9A), 11 (pLC11A), 12 (pBR12), 18 (p-alphaH2), and X (pXBR2), are listed in Table 2.

Double In Situ Hybridization

The DNA probes were labeled by nick translation with biotin-11-dUTP (Brigati et al., 1983) and with digoxigenin-11-dUTP (Lichter et al., 1990). FISH on isolated nuclei was performed as follows: Slides containing isolated nuclei were denatured in 70% formamide/2× SSC for 2 min at 80°C and dehydrated in an ethanol series (70%, 90%, 100%). Forty nanograms of each DNA labeled probe was precipitated with 9 μg of salmon sperm DNA and resuspended in 10 μl of 50% formamide/2× SSC/10% dextran sulfate. Probe DNA was denatured at 75°C for 5 min, ice cooled, and immediately applied to the denatured specimens; a coverslip was added and sealed with rubber cement. FISH on tissue sections was performed as previously described (Taruscio et al., 1993): briefly, labeled probe (40 ng/slide) and sonicated salmon sperm (9 μg/slide) were ethanol precipitated together and redissolved in 50% deionized formamide and 50% hybridization buffer (4× SSC, 20% dextran sulfate). The mixture was applied on tissue sections, which were then covered with an 18 × 18 mm coverslip, sealed with rubber cement, and denatured at 76°C for 10 min.

Nuclei and tissue sections were then incubated in a moist chamber overnight at 37°C. Posthybridization washing was at 42°C in 2× SSC-50% formamide (3 × 5 min), followed by three washes in 0.1× SSC at 60°C. The biotinylated probe was detected using FITC-conjugated avidin DCS (5

TABLE 2. DNA (Peri)centromeric Probes Utilized

Chromosome	Probe name	Reference
3	p3–5	Waye and Willard (1989)
7	p7tet	Waye et al. (1987b)
9	pMR9A	Rocchi et al. (1991)
11	pLC11A	Waye et al. (1987a)
12	pBR12	Baldini et al. (1990)
18	p-alphaH2	Baldini et al. (unpublished data)
X	pXBR2	Yang et al. (1982)

μg/ml; Vector Laboratories), and the digoxigenin labeled probe using anti-digoxigenin was conjugated to rhodamine (3 μg/ml; Boehringer Mannheim). Total DNA was counterstained with DAPI. Triple color images were collected using a computer-controlled Zeiss Axioscope epifluorescence microscope equipped with a cooled charge-coupled device (CCD) camera (Photometrics). FITC, rhodamine, and DAPI fluorescences were recorded separately as gray scale images, and then pseudocolored and merged using a software program developed by T. Rand in D.C. Ward's laboratory.

Sample Examination

FISH on isolated nuclei and tissue sections was examined by 2 independent investigators. Double FISH allowed the simultaneous detection of 2 different target sequences per nucleus. For each probe and sample, 100 intact and nonoverlapping nuclei were randomly selected, and the number of bright, fluorescent spots per nucleus was counted (Van Dekken et al., 1990a,b). Hybridization showed a high efficiency, as in all samples a very small number of nuclei without fluorescent signals were observed; therefore, we did not include the few negative nuclei in the final FISH data (Hopman et al., 1991). In the 2 nodular hyperplasia cases (internal control), we calculated the percentage of nuclei with 1, 2, and 3 hybridization spots for each of the 6 autosomes and the X chromosome, obtaining a total number of samples of n = 14. We then calculated the mean and SD of the percentage of 1 and 3 hybridization spots. A conservative approach was applied; we considered a true monosomy or true trisomy to be present when 1 or more chromosomes exceeded the mean + 4SD. Accordingly, a monosomy was defined when the frequency of single spots was >11% and a trisomy when the frequency of triple spots was >4%. Tetrasomy was almost absent in the nodular hyperplasia tissues; thus 4% was arbitrarily taken as the cutoff value.

RESULTS

FISH on Fresh Isolated Nuclei

Specific (peri)centromeric DNA probes hybridized on nuclei isolated from nodular hyperplasias (considered as internal control) displayed an average percent (±SD) presence of single and triple spots of 4.0 ± 1.7 and 0.43 ± 0.85, respectively (Table 3). The pattern of hybridization observed on the follicular adenoma showed no significant aberrations. Borderline monosomy 18 (12%) was detected in the Hürthle cell adenoma (Table 4).

Table 5 displays the FISH results on the 4 papillary carcinomas with the histological features of the so-called follicular variant. All neoplasms showed at least 2 trisomies. In case 7, all examined autosomes showed trisomies: The most noticeable were for chromosomes 3 (16%), 11 (80%), 12 (72%), and 18 (30%). In the other 3 neoplasms, the most apparent trisomies were for chromosomes 12 (20%) in case 5 and 9 (17%) in case 8. Moreover, 2

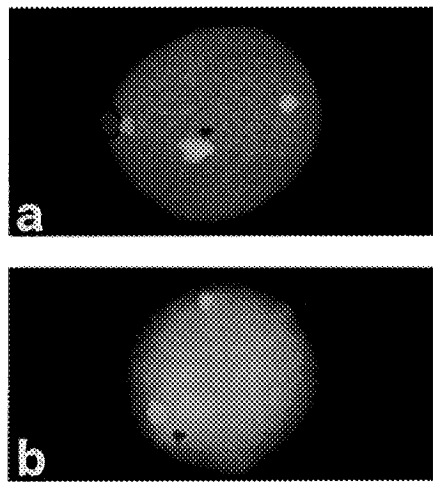

Figure 1. Photomicrographs showing FISH with biotinylated and digoxigenin-labeled chromosome-specific centromeric repeat probes to fresh nuclei isolated from thyroid tumors. The biotinylated probes were detected by fluoresceinated avidin (yellow spots); the digoxigenin-labeled probes were identified by rhodamine (red spots). All DNA was counterstained with DAPI (blue). **a:** Nucleus from papillary carcinoma, follicular variant (case 7). Centromeres of chromosome 9 (red) and chromosome 11 (yellow) are shown. Note trisomy of chromosome 11. **b:** Nucleus from papillary carcinoma, classic type (case 11). Red represents chromosome 9 (note monosomy), whereas yellow is chromosome 7.

TABLE 3. Results of Fluorescence In Situ Hybridization (FISH) With a Set of 7 Chromosome-Specific (Peri)centromeric DNA Probes on Isolated Nuclei From Thyroid Nodular Hyperplasia (Cases 1 and 2)

Probe for chromosome	Number of spots/nucleus							
	Case 1				Case 2			
	1	2	3	4	1	2	3	4
3	6	94	0	0	3	95	2	0
7	3	97	0	0	5	93	2	0
9	8	92	0	0	4	94	2	0
11	2	98	0	0	3	96	0	1
12	4	96	0	0	3	97	0	0
18	2	98	0	0	5	95	0	0
X	5	95	0	0	3	97	0	0

TABLE 4. Results of FISH on Isolated Nuclei From Thyroid Adenomas (Cases 3 and 4)

Probe for chromosome	Number of spots/nucleus							
	Case 3 (follicular adenoma)				Case 4 (Hürthle cell adenoma)			
	1	2	3	4	1	2	3	4
3	5	91	4	0	11	89	0	0
7	3	95	0	2	7	90	3	0
9	6	94	0	0	6	94	0	0
11	4	96	0	0	2	98	0	0
12	6	94	0	0	2	92	2	4
18	6	94	0	0	12	88	0	0
X	6	94	0	0	5	95	0	0

TABLE 5. Results of FISH on Isolated Nuclei From Thyroid Papillary Carcinomas, Follicular Variant (Cases 5–8)

Probe for chromosome	Number of spots/nucleus							
	Case 5				Case 6			
	1	2	3	4	1	2	3	4
3	8	86	6	0	3	94	3	0
7	5	90	5	0	18	82	0	0
9	5	92	0	0	4	86	2	8
11	6	92	0	2	4	94	2	0
12	4	72	20	0	8	84	8	0
18	2	92	6	0	8	82	8	2
X	6	94	0	0	96	0	0	0
	Case 7				Case 8			
	1	2	3	4	1	2	3	4
3	0	78	16	4	9	87	7	1
7	6	79	8	7	4	94	2	0
9	12	69	12	7	7	77	17	9
11	1	14	80	5	3	96	1	0
12	6	15	72	7	5	92	3	0
18	5	56	30	9	6	94	0	0
X	98	0	0	0	97	0	0	0

monosomies were observed, namely, monosomy 7 (18%) in case 6 and monosomy 9 (12%) in case 7; the last tumor was the most aneuploid one in the whole series. Finally, scattered instances of tetrasomies >4% to <10% were observed, mostly in case 7. Tetrasomy of chromosome 9 was observed in 3 out of the 4 papillary carcinomas, follicular variant.

The hybridization pattern of the papillary carcinomas, classical type, showed ≥2 minor (≤10%) trisomies in cases 9 and 10. Chromosomes 11 and 12 were affected in both instances. Trisomy 9 was unexpectedly detected also in the surrounding normal tissue of case 10. On the other hand, a striking monosomy for chromosome 9 (85%) was the sole numerical aberration observed in case 11 (Table 6).

TABLE 6. Results of FISH on Isolated Nuclei From Papillary Carcinomas, Classical Type (Cases 9–11), Including Samples of Normal Surrounding Thyroid Tissue (Cases 10 and 11)

Probe for chromosome	Number of spots/nucleus							
	Case 9							
	1	2	3	4				
3	8	90	2	0				
7	6	92	2	0				
9	10	90	0	0				
11	4	90	6	0				
12	4	86	10	0				
18	10	90	0	0				
X	2	98	0	0				
	Case 10 (normal)				Case 10 (tumor)			
	1	2	3	4	1	2	3	4
3	7	91	2	0	8	86	6	0
7	7	97	2	0	4	91	3	2
9	5	86	9	0	7	83	10	0
11	2	97	1	0	2	92	6	0
12	2	98	0	0	4	90	6	0
18	2	98	0	0	3	91	5	1
X	3	97	0	0	4	96	0	0
	Case 11 (normal)				Case 11 (tumor)			
	1	2	3	4	1	2	3	4
3	5	95	0	0	8	90	2	0
7	6	93	1	0	4	95	1	0
9	1	99	0	0	85	15	0	0
11	5	92	3	0	10	90	0	0
12	4	96	0	0	8	90	1	1
18	2	98	0	0	8	92	0	0
X	4	96	0	0	2	98	0	0

FISH on Frozen Tissue Sections

Table 7 illustrates the comparison between FISH results obtained on 100 isolated nuclei and on 100 apparently nonoverlapping nuclei in tissue sections of the same case. The 2 approaches did not yield homogeneous numbers of hybridization signals. Each probe presented "false" monosomies on sections, presumably due to the cut-effect,

TABLE 7. Comparison Between the Results of FISH With 2 Chromosome-Specific, Alpha-Satellite DNA Probes Applied on Fresh Isolated Nuclei as well as on Frozen Tissue Sections From Case 1 (Nodular Hyperplasia); Case 3 (Follicular Adenoma); Cases 5 and 6 (Papillary Carcinomas, Follicular Variant)

Probe for chrom.	Isolated nuclei/ tissue sections	Number of spots/nucleus							
		Case 1				Case 3			
		1	2	3	4	1	2	3	4
7	Nuclei	3	97	0	0	3	95	0	2
7	Sections	23	77	0	0	4	84	11	1
11	Nuclei	2	98	0	0	4	96	0	0
11	Sections	14	85	1	0	23	66	11	0
		Case 5				Case 6			
		1	2	3	4	1	2	3	4
7	Nuclei	5	90	5	0	18	82	0	0
7	Sections	14	80	4	2	18	75	7	0
11	Nuclei	6	92	0	2	4	94	2	0
11	Sections	22	66	15	0	20	69	11	0

compared with the corresponding hybridization pattern obtained on isolated nuclei. On the other hand, "false" trisomies were also observed (see, e.g., chromosome 11 in case 5), presumably because of the overlapping of the nuclei.

DISCUSSION

In order to detect numerical chromosome aberrations in thyroid neoplasms, we applied double FISH on fresh isolated interphase nuclei and frozen tissue sections obtained from 2 adenomas and 7 papillary carcinomas. We used a set of 7 repetitive (sub)centromeric DNA probes, specific for chromosomes 3, 7, 9, 11, 12, 18, and X. The validity of detecting specific numerical chromosomal aberrations by FISH depends on the hybridization efficiency (Cremer et al., 1988b; Devilee et al., 1988; Hopman et al., 1988; Van Dekken et al., 1989, 1990a,b). The hybridization efficiency can be affected by a low penetration of the probe into the nuclei and by a loss of nuclear DNA. Taking into account this possibility, we used double FISH experiments to have the second probe simultaneously hybridized on the same sample and detected with a different fluorochrome as internal control.

No marked numerical aberrations were found in benign neoplasms, besides a borderline monosomy 18 in the Hürthle cell adenoma, a neoplastic lesion whose fully benign nature is still a matter of debate (Carcangiu et al., 1991).

The follicular variants of papillary carcinomas were the tumors with the highest number of numerical chromosome aberrations. All cases showed trisomies. A specific pattern was not readily identifiable; however, taking into account both the case prevalence and the percentage of affected nuclei, chromosomes 7 and 12 showed the lowest and highest degree of trisomy, respectively. In case 7, all 6 autosomes examined showed trisomies (up to 80% and 72% for chromosomes 11 and 12, respectively); tetrasomies were also present, as was borderline monosomy of chromosome 9. This highly aneuploid tumor did not have any particular histological or clinical features.

In contrast, the classical papillary carcinomas showed variable changes. Two cases (9 and 10) showed several minor ($\leq$10%) trisomies; it is worth noting that chromosome 12 was affected in both instances. In case 11, we observed a severe (85%) monosomy 9. This neoplasm was the only one among the papillary tumors which had metastases: 7 out of 18 lymph nodes examined were positive for carcinoma and lung metastasis was detected radiologically.

Five out of 7 cases of papillary carcinoma altogether displayed trisomy 12, although to a very different extent. One might speculate about the involvement of well-known oncogenes, present on this chromosome, such as *KRAS* (Lemoine et al., 1988). Finally, in the normal tissue surrounding the tumor in case 10, we detected almost the same degree of trisomy 9 as in the neoplastic tissue (9% vs. 10%). Thyroid samples were selected as tumor-free tissue when neoplastic infiltrations were not detected by conventional histological procedures. However, the presence of very small neoplastic infiltrations, involving some more aggressive cellular clones, cannot be completely ruled out.

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